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Wounding of Arabidopsis leaves causes a powerful but transient protection against *Botrytis* infection

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Summary

Physical injury inflicted on living tissue makes it vulnerable to invasion by pathogens. Wounding of *Arabidopsis thaliana* leaves, however, does not conform to this concept and leads to immunity to *Botrytis cinerea*, the causal agent of grey mould. In wounded leaves, hyphal growth was strongly inhibited compared to unwounded controls. Wound-induced resistance was not associated with salicylic acid-, jasmonic acid- or ethylene-dependent defence responses. The phytoalexin camalexin was found to be involved in this defence response as camalexin-deficient mutants were not protected after wounding and the *B. cinerea* strains used here were sensitive to this compound. Wounding alone did not lead to camalexin production but primed its accumulation after inoculation with *B. cinerea*, further supporting the role of camalexin in wound-induced resistance. In parallel with increased camalexin production, genes involved in the biosynthesis of camalexin were induced faster in wounded and infected plants in comparison with unwounded and infected plants. Glutathione was also found to be required for resistance, as mutants deficient in γ-glutamylcysteine synthetase showed susceptibility to *B. cinerea* after wounding, indicating that wild-type basal levels of glutathione are required for the wound-induced resistance. Furthermore, expression of the gene encoding glutathione-*S*-transferase 1 was primed by wounding in leaves inoculated with *B. cinerea*. In addition, the priming of MAP kinase activity was observed after inoculation of wounded leaves with *B. cinerea* compared to unwounded inoculated controls. Our results demonstrate how abiotic stress can induce immunity to virulent strains of *B. cinerea*, a process that involves camalexin and glutathione.

Keywords: induced resistance, priming, *Botrytis cinerea*, wounding, glutathione, camalexin.

Introduction

Botrytis cinerea is an ubiquitous pre- and post-harvest necrotrophic pathogen with a broad host range that causes substantial crop losses (Prins *et al.*, 2000). *Botrytis cinerea* conidia penetrate through the cuticle and epidermal walls, leading to the death of invaded cells. The mycelium spreads through dead tissue, leading to tissue softening, rot or necrosis, depending on the invaded parts (Pezet *et al.*, 2004).

Botrytis circumvents plant defences in various ways. For instance, it can degrade the cuticle and the plant cell wall (Commenil *et al.*, 1998; ten Have *et al.*, 1998; Staples and Mayer, 1995) and detoxify plant anti-fungal products (Gil-ad *et al.*, 2000; Kliebenstein *et al.*, 2005; Pezet *et al.*, 2004; Schoonbeek *et al.*, 2001). During the infection process, *B. cinerea* weakens or damages its host using reactive oxygen species (Govrin and Levine, 2000; Liu *et al.*, 1998; Prins *et al.*, 2000), toxins or oxalic acid, which chelates cell-wall calcium and enhances its own endopolygalacturonase activity (Prins *et al.*, 2000; Reino *et al.*, 2004). Targeted inactivation of fungal genes involved in cell-wall degradation (ten Have *et al.*, 1998; Kars *et al.*, 2005; Valette-Collet *et al.*, 2003) supports the biological relevance of the cell wall as a food source or a general barrier against invading fungi. Further defence reactions induced during infection with *B. cinerea* involve cell-wall strengthening (McLusky *et al.*, 1999; Stewart and Mansfield, 1985).

The defence reactions of the host plant to infection by *B. cinerea* have been studied in many plants. Treatments with oligomers of galacturonic acid protect grapevine against *B. cinerea*, presumably by eliciting defence responses such as increased chitinase and β-1,3-glucanase activities or the stimulation of an oxidative burst (Aziz *et al.*, 2004). Changes in wyerone and wyeronic acid measured in

Vicia faba were found to restrict the growth of *B. cinerea* in broad bean (Mansfield and Hutson, 1980). Accumulation of the lettuce phytoalexin lettucenine correlates with resistance to *B. cinerea* (Bennett *et al.*, 1994). Resistance to *B. cinerea* in Arabidopsis was recently found to involve the phytoalexin camalexin (Denby *et al.*, 2004; Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). The *pad2-1* and *pad3-1* mutants that were isolated based on reduced accumulation of camalexin after inoculation with bacterial pathogens (Glazebrook and Ausubel, 1994) show higher susceptibility to *B. cinerea*, and camalexin was found to have a direct toxic effect against *B. cinerea* (Denby *et al.*, 2004; Ferrari *et al.*, 2003). Earlier observations did not support such a conclusion (Thomma *et al.*, 1999b), but different *B. cinerea* isolates were shown to differ in their camalexin tolerance (Kliebenstein *et al.*, 2005). The production of camalexin was also found to vary greatly among Arabidopsis ecotypes (Denby *et al.*, 2004). This could explain the contradictory results among the various studies.

In Arabidopsis, distinct defence signalling pathways operate against discrete groups of pathogens. For instance, the SA-dependent pathway controls defence to biotrophic pathogens such as *Hyaloperonospora parasitica* and the expression of pathogenesis-related genes such as *PR1* (Nawrath and Métraux, 1999). The ethylene (ET)- and jasmonic acid (JA)-dependent pathways establish a basic level of defence against necrotrophs and control the expression of another set of *PR* genes that include the anti-microbial *PR-3* (encoding chitinase) and *PR-4* (encoding a hevein-like protein) and a plant defensin (PDF1.2) (Thomma *et al.*, 1998). Resistance of tomato to *B. cinerea* is enhanced by ET treatments, and an inhibitor of ET perception increases susceptibility (Diaz *et al.*, 2002). A functional ET pathway is required for a basal level of defence against *B. cinerea* in Arabidopsis (Thomma *et al.*, 1999a). Arabidopsis plants over-expressing *ETHYLENE RESPONSE FACTOR 1*, a gene involved in the ET-dependent expression of proteins such as chitinase or defensins, exhibit increased resistance to necrotrophs (Berrocal-Lobo *et al.*, 2002). Recently, Ferrari *et al.* (2003) proposed that local resistance to *B. cinerea* requires ET, JA and SA signalling pathways, as well as synthesis of camalexin.

Abiotic stimuli such as UV radiation, heat treatment, ozone or wounding have also been reported to be effective inducers of defences against pathogens (Francia *et al.*, 2007; Métraux and Durner, 2004; Schirra *et al.*, 2000; Sharma *et al.*, 1996; Terry and Joyce, 2004). Wounding of the plant surface creates a potential entry point for invading pathogens, and plants respond to this injury by localized defence responses including the induction of defence-related genes (Reymond *et al.*, 2000) and the accumulation of anti-microbial proteins such as proteinase inhibitors or chitinase (Chang *et al.*, 1995; Ryan, 1990). Genetic evidence demonstrates the involvement of octadecanoic acids as endogenous regulators for wound-induced resistance to herbivores (Howe, 2004). However, a substantial number of wound-induced genes are expressed independently of JA perception (Nishiuchi *et al.*, 1997; Reymond *et al.*, 2000; Titarenko *et al.*, 1997). Wounding and insect feeding also produce signals (hydraulic, electrical or chemical) that can propagate systemically. For example, in tomato, wounding results in the production of systemin, a systemic signal involved in the activation of proteinase inhibitors (Ryan and Pearce, 2003). Green leafy volatiles or isoprenoids are produced after mechanical wounding or pathogen/herbivore attacks in higher plants. They are perceived by JA-dependent and -independent pathways in Arabidopsis. Treatment of Arabidopsis plants with such volatiles induces defence responses and increases resistance to *B. cinerea* (Kishimoto *et al.*, 2005).

Many studies have shown that wounding facilitates infection by necrotrophic pathogens as it provides nutrients and creates necrotic areas that may facilitate pathogen ingress in the tissue. Pricking or wounding is often used to inoculate this kind of pathogen into leaves or fruits in laboratory experiments. In this paper, we describe strong immunity of wounded Arabidopsis leaves in response to inoculation with *B. cinerea*. Our experiments have shown priming of camalexin accumulation and of the genes involved in its biosynthesis, and of glutathione-*S*-transferase 1 expression and MAP kinase activity after wounding. The immunity described here highlights a novel defence response of plants, induced by abiotic stress, that bypasses major plant defence pathways involving SA, JA or ET, but requires a wild-type level of glutathione.

Results

Wounding induces a strong immunity against the necrotrophic fungus B. cinerea

Under the experimental conditions described, *B. cinerea* strain BMM was strongly pathogenic on Arabidopsis ecotype Col-0 as shown in Figure 1(a). Wounding of Arabidopsis leaves with laboratory forceps and subsequent inoculation of the wound site with *B. cinerea* led to strong immunity compared to unwounded leaves (Figure 1a). The wound-induced protection could also be induced by puncturing several holes with a syringe needle. No outgrowing lesions were observed after placing a droplet of *B. cinerea* spore suspension on such a wound site (Figure 1a). A single puncture performed with the tip of a syringe needle was not sufficient to induce resistance against *B. cinerea*, and full protection was obtained when inoculation droplets were placed at sites with four punctures or more (data not shown). Inoculation of *B. cinerea* spores distally from a wound site produced symptoms comparable to those on non-wounded leaves, indicating the absence of systemic wound-induced protection (Figure 1b). We complemented these observations on the development of symptoms by following the

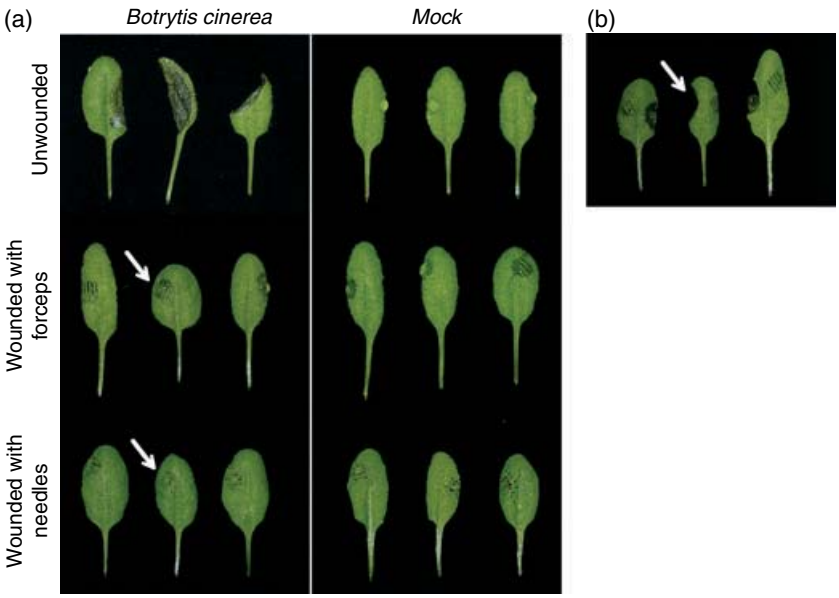

Figure 1. Wound-induced resistance of Arabidopsis against *Botrytis cinerea*.
(a) Disease symptoms on Col-0 WT plants 3 days after mock treatment (right) and inoculation with *B. cinerea* (left). Plants were inoculated by depositing 5 μl of spore suspension on unwounded leaves (top), directly after wounding with a forceps (middle), or directly after puncturing several tiny holes with a syringe needle (bottom). The lesions indicated by the arrows are caused by the wounding.
(b) Leaves of WT plants were wounded with a forceps on one side of the central vein. *Botrytis cinerea* spores (5 μl droplets) were placed on the wounded site and on the unwounded leaf half (arrow). Symptoms were evaluated 3 days after inoculation.

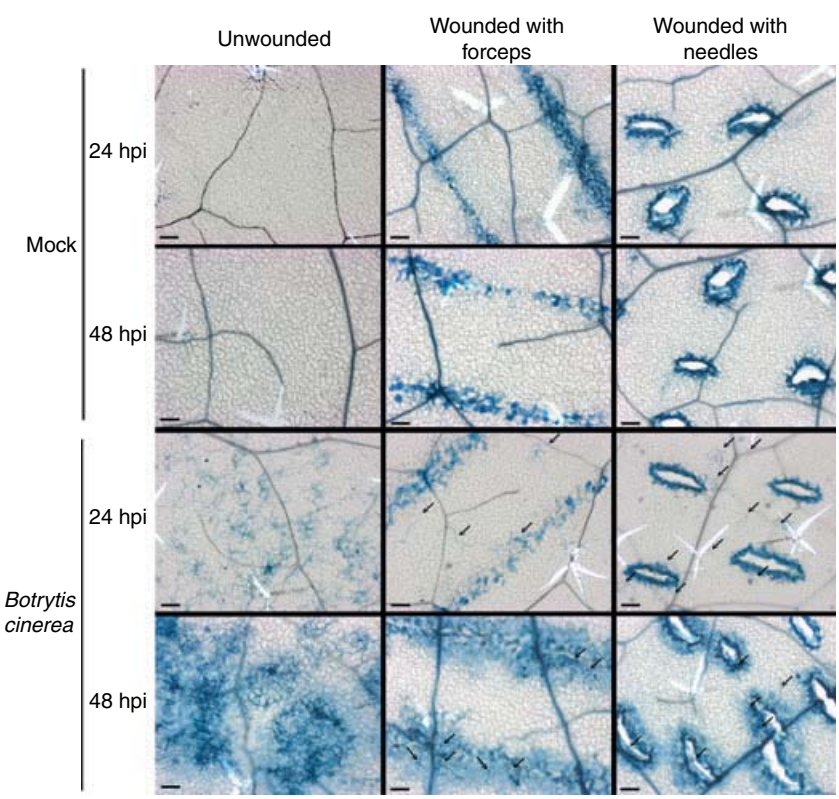

Figure 2. Growth of *Botrytis cinerea* on wounded leaves.
Fungal growth and plant cell death were monitored by staining the leaves of Col-0 WT plants with trypan blue. Wounded and unwounded leaves were harvested 24 and 48 h after mock-inoculation or inoculation with *B. cinerea*. Plants were inoculated by depositing 5 μl of spore suspension or inoculation medium on unwounded leaves (left), directly after wounding with a forceps (middle), or directly after puncturing several tiny holes with a syringe needle (right). Arrows indicate fungal growth in wounded leaves infected with *B. cinerea*. Bars = 20 μm. These images are representative of 20 stained leaves from six to eight plants. The experiments were repeated twice with similar results.

growth of *B. cinerea* using microscopic observations of trypan blue-stained leaves. At wounded sites (needles or forceps), there was little hyphal growth of *B. cinerea* compared to unwounded infected sites at 24 or 48 h post-inoculation (Figure 2), confirming the macroscopic observations shown in Figure 1. Interestingly, the dark blue staining of plant cells observed at or around the wound sites reflects the death of plant cells. However, these dead cells did not facilitate the invasion of tissue by *B. cinerea* (Figure 2).

The level of protection was estimated by the potential of *B. cinerea* to cause soft rot symptoms extending beyond the inoculation site. On wild-type (WT) Col-0 plants, most inoculation sites resulted in water-soaked lesions, but a limited number of lesions did not spread beyond the primary

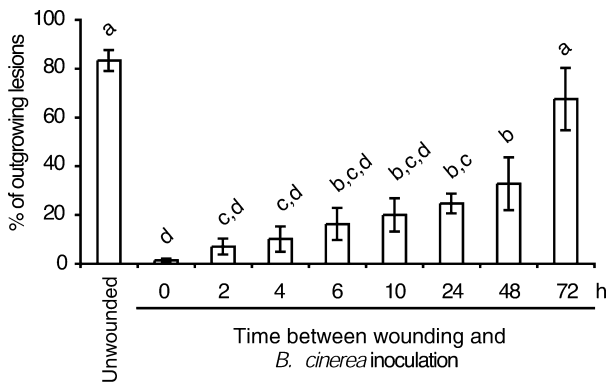

Figure 3. Effect of wound age on wound-induced resistance of *A. thaliana* to *Botrytis cinerea*.
The resistance to *B. cinerea* was determined at various times after wounding and expressed as the percentage of outgrowing lesions relative to the total number of inoculation sites. Plants were wounded with a lab forceps (time 0 h) and inoculated with *B. cinerea* at the times indicated. Symptoms were evaluated 3 days after infection. Bars represent the mean of three to five experiments (±SD). Bars with different letters are significantly different from one another as determined using Duncan's test ($P = 0.05$).

infection site. Under our experimental conditions, the percentage of spreading (outgrowing) lesions was the most suitable way to compare the level of resistance of different plants. All observations with *B. cinerea* strain BMM were repeated with *B. cinerea* strain B05.10 with similar results (data not shown).

Maximum protection was observed when *B. cinerea* spores were inoculated onto the wound site directly after wounding (Figure 3). Nevertheless, if plants were inoculated 24 or 48 h after wounding, outgrowing lesions were observed at 24.7 ± 4.1 and 32.8 ± 10.8% of wound sites, respectively, indicating that 58.7 and 50.6% of the wound sites were protected against *B. cinerea* in comparison to unwounded and infected leaves. However, on a 72 h-old wound site, resistance was barely detectable.

Wound-induced resistance is independent of SA-, JA- and ET-dependent signalling pathways

The dependence of wound-induced resistance on known signalling pathways for induced resistance was tested using several mutants as shown in Figure 4(a). Wounding was performed on mutants affected in ET perception (such as *ein2* and *etr1*), JA signalling (*jar1*, *coi1*), SA accumulation (*sid2*, *pad4*) or in the triple mutant *nej* (*npr1*, *ein2*, *jar1*) that is blocked in SA, JA and ET perception (Clarke *et al.*, 2000). Inoculation of the wound sites with *B. cinerea* induced resistance in all mutants to the same extent as in WT plants, indicating that ET, JA and SA signalling are not involved in the wound-induced resistance.

Wound-induced resistance depends on camalexin production

Furthermore, the phytoalexin-deficient mutants *pad2*, *pad3* (Glazebrook and Ausubel, 1994) and *ups1* (Denby *et al.*, 2005) were tested, as camalexin has previously been implicated in resistance to *B. cinerea* (Denby *et al.*, 2004; Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). Interestingly, protection induced by wounding was much less efficient in both *pad3* and *ups1* (Figure 4a) and almost totally suppressed in *pad2* (Figure 4b) compared to WT plants. These results indicate that camalexin is involved in the wound-induced resistance to *B. cinerea.* In a recent study, camalexin was shown to have a variable impact on *B. cinerea*, depending on the pathogen isolate (Kliebenstein *et al.*, 2005). Therefore, the sensitivity of the *B. cinerea* isolates BMM and B05.10 used here was tested. *In vitro* tests in which camalexin was incorporated in the germination medium confirmed a strong sensitivity of *B. cinerea* strains BMM and B05.10 to camalexin (Table 1). The sensitivity of the strains used in this study was similar to that reported for other strains described as sensitive in a previous study (Kliebenstein *et al.*, 2005).

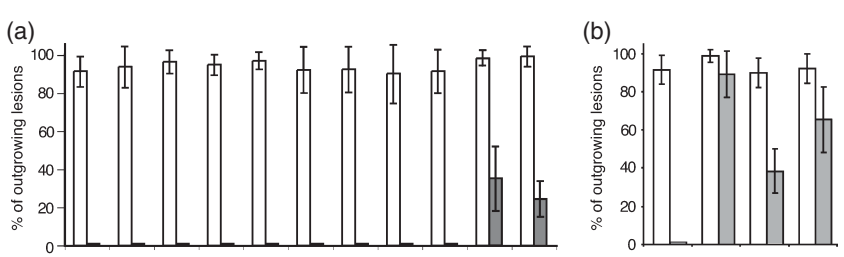

Figure 4. Wound-induced resistance to *Botrytis cinerea* in various *A. thaliana* mutants.
(a) Signalling and camalexin mutants. The resistance level of WT plants and various mutants to *B. cinerea* was evaluated as the percentage of outgrowing lesions relative to the total number of inoculation sites. White bars, unwounded leaves; grey bars, leaves wounded with forceps. Symptoms were evaluated 3 days after inoculation. Bars represent the means of two to six experiments (±SD).
(b) GSH1 mutants. The resistance level of WT plants and various GSH1 mutants to *B. cinerea* was evaluated as the percentage of outgrowing lesions relative to the total number of inoculation sites. White bars, unwounded leaves; grey bars, leaves wounded with forceps. Symptoms were evaluated 3 days after infection, and the data are the means of three independent experiments performed on 24 plants for each genotype (±SD).

Table 1 Effect of camalexin on *Botrytis cinerea* growth on liquid medium

	Botrytis cinerea strain	
	B05.10	BMM
EC_{50}	4.8 ± 0.9	4.2 ± 1.1
EC_{95}	22.4 ± 4.3	15.1 ± 7.1

EC_{50} and EC_{95}: 50 and 95% effective concentration (mg l^{-1}) ± experimental error, respectively.

To further explore the importance of camalexin, the kinetics of camalexin accumulation in wounded WT plants after inoculation with *B. cinerea* were determined (Figure 5a). No camalexin accumulated after spraying inoculation medium on wound sites. The accumulation of camalexin in WT plants was triggered at 24 h after *B. cinerea* inoculation and increased at 36 h post-inoculation (hpi). The highest levels of camalexin were observed at 48 hpi in inoculated unwounded plants due to the extensive disease development in the absence of wounding. Similar observations have been made by Kliebenstein *et al.* (2005). These authors showed that camalexin accumulates at high levels at the infected site and immediately adjacent areas following inoculation with *B. cinerea*. Interestingly, in wounded and infected plants, camalexin was already detectable at 12 hpi. At 24 hpi, it rose to a level sevenfold higher than that in unwounded *B. cinerea*-inoculated plants (Figure 5a).

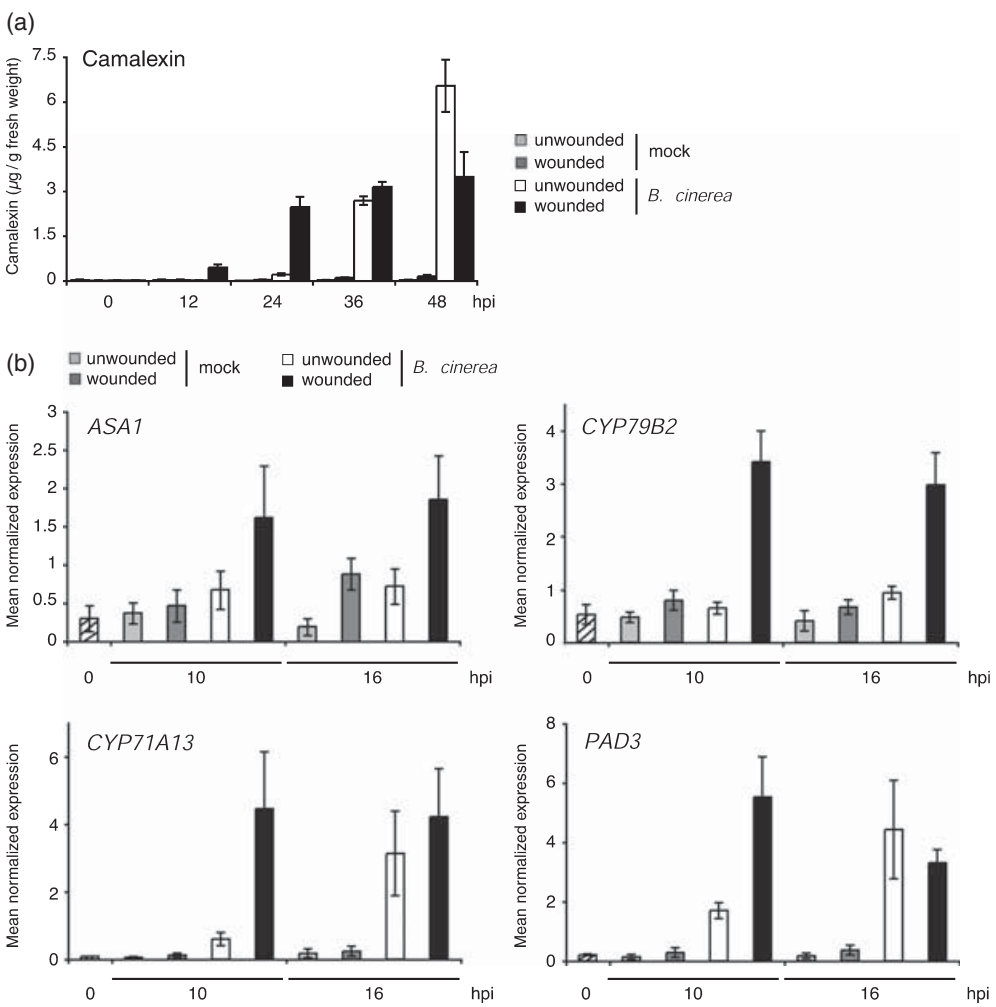

Figure 5. Priming of camalexin accumulation and induction of camalexin biosynthetic genes in wounded and inoculated *A. thaliana* Col-0 plants.
(a) Priming of the accumulation of camalexin. Wild-type Col-0 plants were sprayed with the mock buffer (light grey bars), wounded and mock-sprayed (dark grey bars), sprayed with *B. cinerea* (white bars), or wounded and inoculated (black bars). Wounding was performed by puncturing the whole leaf surface with the syringe 'needle stamp'. Three replicates of leaves from at least six plants were collected at various time points after treatment. Camalexin was extracted and quantified by HPLC by comparing peak areas with camalexin standards. Data are the means of the three replicate samples (±SD). The experiment was repeated twice with similar results.
(b) Priming of the induction of camalexin biosynthetic genes. Plant treatment and wounding were performed as described in (a). The transcript levels of *ANTHRANILATE SYNTHASE 1* (*ASA1*), *CYP79B2*, *CYP71A13* and *PAD3* were quantified by real-time PCR and normalized to the transcript level of the plant reference gene *At4g26410* (Czechowski *et al.*, 2005). Hatched bars indicate the level of gene expression in untreated plants. Data are expressed as mean normalized expression (no units) and are the means of duplicates (±SD). The experiment was repeated twice with similar results.

Therefore, wounding predisposes (primes) the tissue for faster production of camalexin after *B. cinerea* inoculation compared to unwounded controls. The molecular basis for camalexin accumulation after wounding and infection with *B. cinerea* was further studied using DNA microarrays. Four genes related to the biosynthesis of camalexin showed an increased induction in wounded and *B. cinerea*-infected plants compared to unwounded and infected plants. These genes included those encoding anthranilate synthase 1 (*ASA1*), the cytochrome P450 enzymes *CYP79B2* and *CYP71A13*, and *PAD3* (Glawischnig *et al.*, 2004; Nafisi *et al.*, 2007; Schuhegger *et al.*, 2006, 2007; Zhou *et al.*, 1999). These data were confirmed by real-time RT-PCR analyses. A stronger induction of these four genes was already detected at 10 hpi in wounded and infected leaves compared to unwounded and infected leaves (Figure 5b), suggesting that the priming of camalexin accumulation observed in wounded and infected plants is based on primed transcriptional activation of camalexin biosynthetic genes. The primed induction of the genes involved in camalexin accumulation (already visible at 10 hpi) preceded accumulation of camalexin (visible at 12 and 24 hpi, Figure 5a).

The wound-induced priming of camalexin was further analysed in the mutants of the ET (*ein2*), JA (*jar1*) and SA (*pad4*) pathways used in the experiment described in Figure 4. Camalexin levels were determined at 24 hpi, the time at which priming has taken place after wounding and inoculation in WT plants (Figure 5a). As shown in Figure 6, strong induction of camalexin was measured in the *ein2*, *jar1* and *pad4* mutants, indicating wound-induced priming similar to that induced in WT plants. *pad3* and *ups1* showed absence or an intermediate level of camalexin accumulation in response to *B. cinerea* in wounded leaves, respectively. The absence of camalexin in *pad3* agrees with published results describing PAD3 as a cytochrome P450 monooxygenase that is directly involved in the last step of camalexin biosynthesis (Schuhegger *et al.*, 2006). Although *pad2* was the most susceptible of all mutants to *B. cinerea* (Figure 4b), it did not display the lowest level of camalexin after wounding and infection at 24 hpi (Figure 6). This suggests the participation of other components in the wound-induced resistance in addition to camalexin.

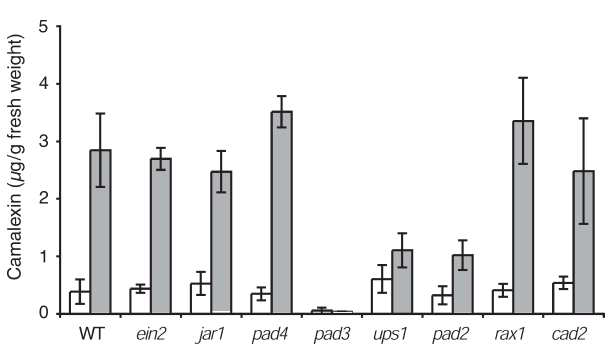

Figure 6. Wound-induced priming of camalexin in *A. thaliana* Col-0 plants and various mutants.
Unwounded (white bars) and syringe-wounded plants (grey bars) were spray-inoculated with *Botrytis cinerea*. Three leaf samples were harvested 24 h after inoculation and camalexin was extracted. Means of three replicates (±SD) are shown.

A basal level of glutathione is a key element controlling wound-induced resistance

The *pad2* mutant has recently been shown to carry a mutation in *GSH1*, which encodes a γ-glutamylcysteine synthetase involved in the biosynthesis of glutathione (Parisy *et al.*, 2007). To confirm the possible role of glutathione, two other glutathione-deficient mutants, *cad2-1* (*cadmium hypersensitive*) and *rax1-1* (*regulator of ASCORBATE PEROXIDASE 2*) were analysed. Both *cad2* and *rax1* carry point mutations in the *GSH1* gene (Ball *et al.*, 2004; Cobbett *et al.*, 1998). Interestingly, *rax1* and *cad2* show a strong reduction in the wound-induced protection to *B. cinerea*, confirming a possible involvement of glutathione in this phenomenon (Figure 4b).

To find out whether a basal level of glutathione is required for the wound-induced resistance to *B. cinerea*, the level of glutathione was determined in Arabidopsis WT plants, in *pad2*, *cad2* and *rax1*, and in *pad3* and *ups1* which were included as susceptible plants (Figure 7). In accordance with published results, the level of glutathione was strongly decreased in *pad2*, *rax1* and *cad2*, compared to WT plants (Ball *et al.*, 2004; Cobbett *et al.*, 1998; Parisy *et al.*, 2007). Among the three *GSH1* mutants, *pad2* had the lowest glutathione level (11.62 ± 1.04 ng/g FW) in comparison to WT Col-0 plants (96.51 ± 5.89 ng/g FW). However, the basal level of glutathione was higher in *cad2* and *rax1* (30.46 ± 1.15 and 23.06 ± 1.04 ng/g FW, respectively) than in *pad2*. Furthermore, and unlike camalexin, neither wound-induced accumulation of glutathione, wound-induced priming of glutathione accumulation nor wound-induced priming of the change in the ratio of reduced to oxidized

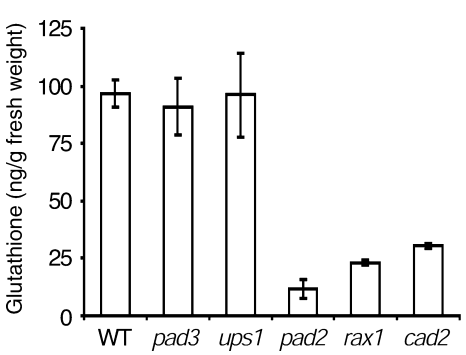

Figure 7. Basal level of glutathione in *A. thaliana* Col-0 plants and various mutants.
Leaves of non-induced 4-week-old plants were analysed. Means (±SD) from three independent measurements are shown.

glutathione concentration could be observed in WT plants (data not shown). All these results indicate that a WT basal level of glutathione is required for wound-induced resistance to *B. cinerea*.

Priming of camalexin accumulation 24 h after wounding and inoculation with *B. cinerea* was strongly decreased in *pad2* (Figure 6). Despite priming of camalexin accumulation in *cad2* and *rax1* similar to that in WT plants (Figure 6), these two mutants were strongly affected in wound-induced resistance (Figure 4b) and showed a low basal level of glutathione (Figure 7). These data indicate that glutathione is involved in other important processes that allow wound-induced resistance in addition to its role in camalexin production.

Glutathione is involved in the detoxification of organic compounds. Many xenobiotics as well as some metabolites such as anthocyanins are conjugated to glutathione by the family of glutathione-*S*-transferases (GST) and transported, possibly as conjugates, into the vacuole (Marrs, 1996). One of these GSTs, GST1 (Greenberg *et al.*, 1994) was selected previously as a robust molecular marker for the production of reactive oxygen species expressed independently of SA, JA and ET after wounding and pathogen attack (Grant and Loake, 2000; Kishimoto *et al.*, 2005; Reymond *et al.*, 2000). *GST1* expression was analysed by real-time RT-PCR after wounding and inoculation with *B. cinerea* in WT plants (Figure 8). *GST1* mRNA accumulation was primed and already detectable at 9 hpi and strongly increased at 15 hpi in wounded and inoculated plants. *GST1* expression was strongly induced by *B. cinerea* alone only after 24 hpi. However, *GST1* was also induced moderately by wounding alone, and expression reached a maximum at 9 hpi and decreased thereafter.

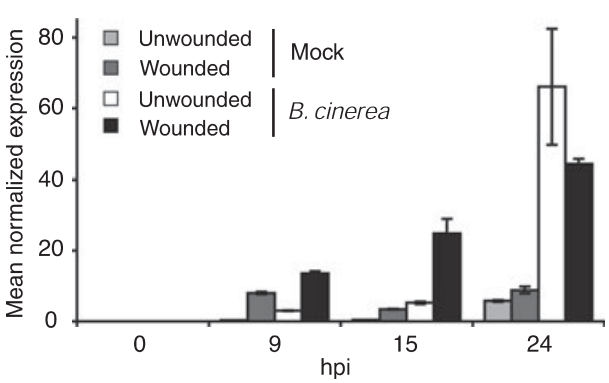

Figure 8. Priming of *GST1* mRNA accumulation.
Wild-type Col-0 plants were mock-sprayed (light grey bars), wounded and mock-sprayed (dark grey bars), sprayed with *Botrytis cinerea* (white bars), and wounded and sprayed with *B. cinerea* (black bars). Wounding was performed using the syringe 'needle stamp'. Leaves from four to six plants were harvested at various time points for each treatment; RNA was extracted and retrotranscribed in cDNAs. The *GST1* transcript level was quantified by real-time PCR and normalized to the transcript level of the plant reference gene *At4g26410* (Czechowski *et al.*, 2005). Data are expressed as mean normalized expression (no units), and are the means of triplicates (±SD). The experiment was repeated twice with similar results.

All the results presented here suggest that WT basal glutathione levels and expression of the stress-inducible *GST1* gene play a role in wound-induced resistance to *B. cinerea*.

Wounding primes MAPK activation in response to B. cinerea

In addition to probing the role of the major defence signalling pathways, the possibility that wound-induced resistance could be the consequence of modification of early signalling events was also examined. Protein phosphorylation signalling modules are important relays between recognition of early stimuli and the activation of defence responses (Asai *et al.*, 2002). Here, wound-induced priming of kinases (Figure S1a and Appendix S1) that corresponded to MPK3 and MPK6 was observed (Figure S1b,c). The priming of MAP kinase activity was detected at a similar time point (10 hpi) to the priming of expression of *GST1* (9 hpi) and of genes for camalexin biosynthesis (10 hpi). To investigate the role of these kinases in wound-induced resistance, the *mpk3* and *mpk6* mutants were tested. Both mutants were protected against *B. cinerea* after wounding (Figure S1d). Because these two kinases have overlapping functions in Arabidopsis (Miles *et al.*, 2005), the use of a double mutant could help to understand their shared function. However, the *mpk3 mpk6* double mutant has been shown to be lethal (Wang *et al.*, 2007).

Discussion

In general, wounding of plant surfaces offers an ideal entry point for many micro-organisms that may invade plants, and a number of pathogenic species use such breaches to invade their host. Plants have evolved mechanisms to recognize and respond to these injuries by activating various resistance mechanisms against micro-organisms or insects (Kuc, 2000; Reymond *et al.*, 2000). The results presented here extend these observations to the case of an infection with the necrotrophic fungus *B. cinerea*. A very strong resistance was observed at the site of wounding (Figures 1 and 2). The wound surface seems to be required to activate resistance responses by offering an increased surface area for induction of stress reactions. Our microscopical observations indicated that cell death associated with wounding did not enhance fungal colonization (Figure 2). This is somewhat in opposition to the classical model indicating that necrotrophic pathogens such as *B. cinerea* utilize plant cell death to their own benefit to invade plant tissue (Glazebrook, 2005; Govrin and Levine, 2000).

In previous studies, basal resistance to *B. cinerea* was mainly associated with ET- and JA-dependent defence responses (Diaz *et al.*, 2002; Thomma *et al.*, 1998, 1999b).

Local resistance to *B. cinerea* was also reported to depend on SA (Ferrari *et al.*, 2003). However, wound-induced resistance to *B. cinerea* is independent of the JA, ET and SA defence pathways, as shown using various mutants (Figure 4a). Our observations extend and contrast with these previous results, and highlight the activation of a strong and localized immunity against *B. cinerea* at wound sites.

Following pathogen ingress, plants produce low-molecular-weight anti-microbial compounds, including phytoalexins (Garcion *et al.*, 2007). In *Arabidopsis thaliana*, the phytoalexin camalexin is produced in response to a wide variety of stimuli, including infection by *B. cinerea*, and several studies have indicated that camalexin is necessary for the defence mechanisms of the plant against this necrotrophic pathogen (Denby *et al.*, 2004; Ferrari *et al.*, 2003; Kliebenstein *et al.*, 2005). The wound-induced resistance to *B. cinerea* described here depends on camalexin as (i) the *B. cinerea* isolates used here are sensitive to camalexin (Table 1), (ii) mutants impaired in camalexin accumulation such as *pad3*, *ups1* and *pad2* are less protected against *B. cinerea* by wounding (Figure 4), (iii) the accumulation of camalexin and the induction of genes involved in its biosynthesis occurs earlier and to a greater extent in wounded and infected plants in comparison with unwounded and infected plants (Figure 5). These observations clearly support an important but partial role for camalexin in the resistance induced by wounding. The faster and stronger camalexin accumulation that takes place in wounded plants upon inoculation with *B. cinerea* (Figure 5a) allows an anticipated reaction against the initial stages of the pathogen development. The earlier and enhanced transcriptional up-regulation of camalexin biosynthetic genes (Figure 5b) is in agreement with other studies that indicate coordinated up-regulation of biosynthetic genes when camalexin is produced (Glawischnig, 2007; Nafisi *et al.*, 2007; Schuhegger *et al.*, 2007). In contrast, induction of camalexin by the pathogen in unwounded conditions is likely to be too slow (Figure 5a), and the pathogen can outgrow the sites of camalexin production. Figure 5(a) shows a higher accumulation of camalexin at 48 h after infection in unwounded compared to wounded plants. However, at this time point, the tissue is already well colonized (Figure 2), and camalexin production is strongly induced by the damage caused by the pathogen. Enhanced phytoalexin accumulation at later time points during compatible plant–pathogen interactions is a well-known phenomenon (Yoshikawa *et al.*, 1978, Hahn *et al.* 1985). Rapid and strong production has long been recognized as critical for phytoalexins to be useful for defence (Yoshikawa *et al.*, 1978; Hahn *et al.* 1985). Importantly, and unlike other metabolites such as sinapyl malate, flavonols, indole or aliphatic glucosides, the accumulation of camalexin in response to *B. cinerea* was confined to the necrotized infection area (Kliebenstein *et al.*, 2005). This localized accumulation of camalexin might explain the absence of systemic resistance in response to localized wounding. Therefore, wound-induced resistance is associated with enhanced mobilization of plant defences. Wounding was found to predispose the defence of the tissue to an infection by a necrotrophic pathogen localized at the wound site. This type of process is generally referred to as priming, and has been described to take place after exposure of plants to chemicals (e.g. β-aminobutyric acid), fungi, bacteria or airborne signals in plant–insect interactions (Conrath *et al.*, 2002; Engelberth *et al.*, 2004).

The complete loss of wound-induced resistance in *pad2* compared to *pad3* (Figure 4a,b), despite a somewhat higher camalexin content in wounded and infected *pad2* leaves compared to *pad3* (Figure 6), implies that wound-induced resistance must involve factors additional to camalexin. One of these factors is glutathione, as *pad2* has recently been shown to carry a mutation in the gene encoding γ-glutamylcysteine synthetase (GSH1), the first enzyme in glutathione biosynthetic pathway (Parisy *et al.*, 2007). This is in agreement with results obtained with two other *GSH1* mutants, *rax1* and *cad2*, that are also strongly impaired in wound-induced resistance to *B. cinerea*. The WT level of glutathione appears to be sufficient for wound-induced resistance, as no increase of glutathione accumulation or priming in wounded and inoculated WT plants was observed (data not shown).

Glutathione is a powerful cellular redox agent. It is involved in almost all major physiological reactions, including development and defence against biotic and abiotic stresses, as well as sulphur, nitrogen and carbon metabolism (Noctor and Foyer, 1998). The results presented here clearly indicate that glutathione is likely to be involved in the priming of camalexin accumulation. Indeed, the *pad2* mutant had the lowest basal level of glutathione (Figure 7), was strongly impaired in the priming of camalexin accumulation (Figure 6), and was the least protected mutant tested so far (Figure 4b) following wounding and infection with *B. cinerea*. Interestingly, expression of the genes involved in camalexin biosynthesis was still primed after wounding and infection in *pad2* plants (data not shown). We conclude that, in wound-induced resistance, glutathione acts on a step downstream of the transcription of biosynthetic genes for camalexin production. Glutathione has been proposed to regulate camalexin biosynthesis or serve as a biosynthetic precursor for the thiazole ring of camalexin (Glawischnig, 2007; Parisy *et al.*, 2007; Schuhegger *et al.*, 2006). The data presented here are in agreement with these views.

In addition to camalexin, glutathione is likely to control other important processes for wound-induced resistance, because *rax1* and *cad2* were strongly impaired in wound-induced resistance (Figure 4b) despite showing priming of camalexin production comparable to wounded and infected WT plants (Figure 6). Although the level of wound-induced

resistance in *pad2*, *rax1* and *cad2* is lower than in WT plants, our results do not show a linear correlation between basal levels of glutathione and resistance (Figures 4b and 7) despite a common defect in GSH1. This may not be so surprising, as *rax1* and *cad2* plants present many differences in the expression profiles of defence and stress genes although they bear a mutation in the same gene (Ball *et al.*, 2004). In addition, glutathione modulates the redox state of thiol groups of proteinaceous redox co-factors, affecting many cellular processes (Ball *et al.*, 2004). Glutathione can also modify regulatory proteins post-translationally by targeting free thiols and inducing S-glutathionylation (Dalle-Donne *et al.*, 2007; Foyer and Noctor, 2005). Glutathione might also participate in the detoxification of phytotoxic compounds (Marrs, 1996). Perhaps plant cells also require glutathione to avoid excessive damage caused by the accumulation of phytoalexins, or to quench the oxidative stress inflicted by the pathogen. The glutathione-*S*-transferase gene *GST1* is used as marker gene for pathogen- and wound-induced responses (Jabs *et al.*, 1996; Kishimoto *et al.*, 2005). As a next logical step, *GST1* expression was tested to determine whether it might be affected by wounding and subsequent infection. Our results show wound-induced priming of *GST1* in infected WT plants (Figure 8). It was also recently shown in the *ups1* mutant that reduced *GST1* expression accompanies lower camalexin accumulation (Denby *et al.*, 2005). In addition, the mutant *ocp3* (*over-expressor of cationic peroxidase 3*) shows increased resistance to *B. cinerea* and constitutively expresses the *GST1* marker gene (Coego *et al.*, 2005). Taken together, the results discussed above indicate that wound-induced resistance to *B. cinerea* might be linked to faster and enhanced expression of *GST1*, possibly due to conjugation of glutathione to toxic compounds (Gil-ad *et al.*, 2000). In conclusion, glutathione represents an essential component of the wound-induced resistance to *B. cinerea*, influencing timely accumulation of camalexin and on other as yet unknown mechanisms.

Our results show priming of MAP kinase activity, which supports the hypothesis of a possible link between early events and the activation of defence responses via protein phosphorylation. Here, wound-induced priming of MAP kinases (Figure S1a) was observed that corresponds to the action of MPK3 and MPK6 (Figure S1b,), two MAP kinases that are induced in plants infected with *B. cinerea* (Veronese *et al.*, 2006). Furthermore, it has been suggested that a MEKK1–MKK4/5–MPK3/6 signalling pathway is involved in resistance of Arabidopsis to *B. cinerea* infection (Asai *et al.*, 2002). The *mpk3* and *mpk6* mutants still exhibited wound-induced immunity (Figure S1d), and the *mpk3 mpk6* double mutant would have been interesting to test, but unfortunately it has been shown to be lethal (Wang *et al.*, 2007). Our results might be explained by an overlapping function for MPK3 and MPK6 (Miles *et al.*, 2005). The link between the priming of MAP kinases and wound-induced immunity thus remains to be established.

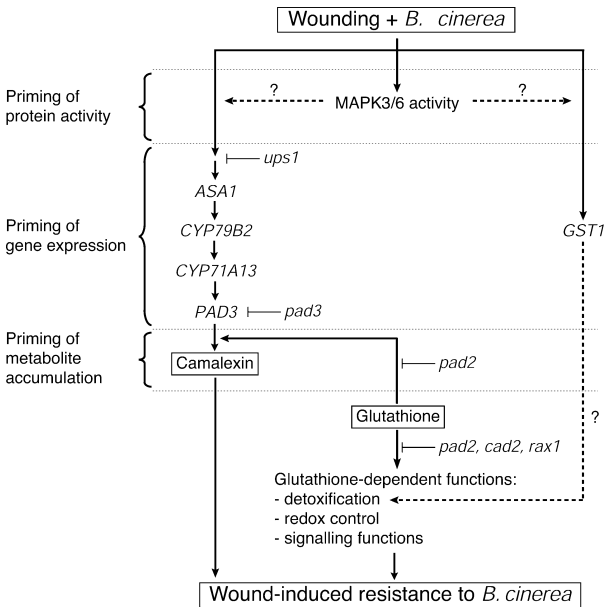

Figure 9. Schematic representation of wound-induced resistance to *Botrytis cinerea*.
This process is fully operational in mutants of the SA pathway (*sid2*, *pad4*), ET (*ein2*, *etr1*) or JA (*jar1*, *coi1*) as well as in the triple mutant of the SA, ET and JA pathways (*nej*). The scheme indicates the importance of the priming of camalexin as well as the effect of glutathione on priming of the accumulation of camalexin and other functions. The inhibitory effects of camalexin and glutathione accumulation are also indicated. The *ups1* mutation is localized upstream of *ASA1* and *PAD3* as described previously (Denby *et al.*, 2005). The possible connection between the observed priming of MAPK3/6 activity and *GST1* expression is also indicated.

Summarizing, wounding of Arabidopsis Col-0 induces a strong immunity to *B. cinerea*. Wound-induced resistance depends on priming of plant defences. Interestingly, the priming was observed at the level of enzyme activity (MAPK), transcription of genes (*ASA1*, *CYP79B2*, *CYP71A13*, *PAD3*, *GST1*) and metabolite accumulation (camalexin) (Figure 9). We have demonstrated that this resistance involves camalexin, a toxic compound against *B. cinerea*, and requires glutathione. This molecule is involved in the priming of camalexin accumulation (metabolite priming) and in other functions, one of which presumably involves GST1. These results add an interesting dimension to our understanding of the perception and reaction of plants to abiotic and biotic stresses.

Experimental procedures

Plant maintenance

Plants were grown on a pasteurized soil mix of humus/perlite (3:1) under a 12 h light/12 h dark cycle, with a night temperature of 16–18°C and a day temperature of 20–22°C (60–70% humidity). WT

plants are the Arabidopsis accession Col-0 obtained from the Arabidopsis Biological Research Center (Columbus, OH, USA). The Arabidopsis mutant referred to as *eds5* was *eds5-3*, *sid2* was *sid2-1*, *pad2* was *pad2-1*, *pad3* was *pad3-1*, *pad4* was *pad4-1*, *etr1* was *etr1-1*, *ein2* was *ein2-1*, *jar1* was *jar1-1* and *coi1* was *coi1-1* as previously described (Chassot *et al.*, 2007; Heck *et al.*, 2003). The mutant referred to as *rax1* was *rax1-1* (Ball *et al.*, 2004) and *cad2* was *cad2-1* (Cobbett *et al.*, 1998).

Culture of B. cinerea *and infection method*

Botrytis cinerea strains B05.10 and BMM, provided by Jan van Kan (Wageningen University, The Netherlands) and Brigitte Mauch-Mani (University of Neuchâtel, Switzerland), respectively, were grown on Difco potato dextrose agar, 39 g l^{-1} (Becton Dickinson, http://www.bd.com). Spores were harvested in water and filtered through glass wool to remove hyphae. Spores were diluted in quarter-strength Difco potato dextrose broth, 6 g l^{-1} (Becton Dickinson) for inoculation. Droplets of 5 μl of spore suspension (5×10^4 spores ml^{-1}) were deposited on leaves of 4-week-old plants for quantification of outgrowing lesions. The spore suspension (2×10^5 spores ml^{-1}) was sprayed on whole plants for camalexin measurements, real-time RT-PCR experiments and MPK analysis. The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Control plants were inoculated with quarter-strength potato dextrose broth.

Wounding method

Wounding was performed using a sharp laboratory forceps or by puncturing several holes (approximately 10) using a syringe needle (27GA). For wounding of whole leaves, a custom-made 'needle stamp' comprising syringe needles 1 mm apart was used. Wounded leaves were inoculated and covered directly (within 10 min) after treatment.

Trypan blue staining

Fungal structures and dead plant cells were stained by boiling inoculated leaves for 1 min in alcoholic lactophenol trypan blue solution. Stained leaves were extensively cleared in chloral hydrate (2.5 g ml^{-1}) at room temperature with gentle shaking, and then observed under a microscope at 5× magnification.

Camalexin determination

Leaf material (approximately 200 mg) was collected and assayed for camalexin as previously described for SA (Heck *et al.*, 2003). Samples were frozen and ground with a glass rod, then 2 ml of 70% v/v ethanol and 200 ng of internal standard (ortho-anisic acid, 1 ng μl^{-1} in ethanol) was added. After homogenization (Polytron, Kinematica, http://www.kinematica-inc.com) and centrifugation (10 min, 10 000 ***g***), the supernatant was decanted into a fresh tube and the extraction was repeated with 2 ml of 90% v/v methanol. Supernatants were pooled, evaporated under reduced pressure and 200 μl of 5% trichloroacetic acid was added to the remaining aqueous solution. After a brief centrifugation (10 min, 10 000 ***g***), the supernatant was transferred to a fresh tube and extracted with 2 × 500 μl of ethyl acetate/cyclohexane (1:1). The pooled organic phases that contain the free phenols and camalexin were evaporated (Speed Vac, Buchler) and resuspended in 200 μl (or more depending on the amount of plant tissue) of HPLC starting buffer (15% acetonitrile in 25 mM KH_2PO_4, pH 2.6). Chromatography was performed on a reverse-phase HPLC column (ABZ+, 25 cm × 4.6 mm, Supelco, http://www.sigmaaldrich.com). The amount of camalexin was calculated in μg g^{-1} fresh weight with reference to the amount of internal standard.

In vitro *effect of camalexin on the growth of* B. cinerea

To establish the sensitivity of *B. cinerea* strains B05.10 and BMM to camalexin, the inhibition of growth in half-strength potato dextrose broth, 12 g l^{-1} was measured in 96-well plates as previously described (Schoonbeek *et al.*, 2001). The 50% (EC_{50}) and 95% (EC_{95}) effective concentration values for inhibition of *B. cinerea* growth were calculated from a series of nine camalexin concentrations ranging from 0.56 to 56 mg l^{-1}.

Glutathione extraction

Samples were extracted as described previously (Parisy *et al.*, 2007). Leaf material (approximately 200 mg) was collected, frozen and ground with a glass rod. HCl (2 ml, 0.1 N) and homoglutathione (50 μg, internal standard) were added before homogenization (Polytron, Kinematica). Samples were centrifuged at 4°C (10 min, 13 000 ***g***), and 120 μl of the supernatant was mixed with 200 μl of CHES buffer (0.2 M 2-*N*-cyclohexylamino-ethane sulphonic acid, pH 9.3). For reduction of total disulphides, 10 μl of BMS (9 mM *bis*-2-mercaptoethylsulphone in 200 mM Tris/HCl and 5 mM EDTA, pH 8.0) were added to the samples for 40 min at room temperature. Free thiols were labelled for 15 min at room temperature in the dark using 15 μl of 15 mM monobromobimane in acetonitrile. The reaction was stopped with 250 μl of 15% HCl. The samples were kept on ice, centrifuged for 10 min at 13 000 ***g***, and analysed by HPLC as described previously (Parisy *et al.*, 2007). The amount of total thiols was calculated in ng g^{-1} fresh weight with reference to the amount of internal standard.

RNA extraction and real-time RT-PCR

RNA was prepared using TRIzol® reagent (Invitrogen, http://www.invitrogen.com/). RNA (1 μg) was retrotranscribed into cDNA (Omniscript® RT kit, Qiagen, http://www.qiagen.com/). Real-time PCR was performed using Absolute QPCR SYBR Green Mix (ABgene, http://www.abgene.com). Gene expression values were normalized to expression of the plant gene *At4g26410*, previously described as a stable reference gene (Czechowski *et al.*, 2005). The primers used were 5′-*At4g26410* (5′-GAGCTGAAGTGGCTTCCATGAC-3′), 3′-*At4g26410* (5′-GGTCCGACATACCCATGATCC-3′), 5′-*GST1* (or *ATGSTF6*) (5′-ATCAAAGTTTTCGGTCACCCA-3′), 3′-*GST1* (5′-TTTACCAAAGGGGTTGCGAAG-3′), 5′-*ASA1* (5′-AACGATGTTGGAAAGGTTACG-3′), 3′-*ASA1* (3′-CGTCCCAGCAAGTCAAACC-3′), 5′-*CYP79B2* (5′-CTCGCGAGACTTCTTCAAGG-3′), 3′-*CYP79B2* (5′-CCATAACCAACGGTTTAGCC-3′), 5′-*CYP71A13* (5′-TAAAGAGGTGCTTCGGTTGC-3′), 3′-*CYP71A13* (5′-TATCGCAGTGTCTCGTTGGA-3′), 5′-PAD3 (5′-TGCTCCCAAGACAGACAATG-3′) and 3′-PAD3 (5′-GTTTTGGATCACGACCCATC-3′).

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